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The Structure and Innervation of the Venom Glands in the Tail of the Salamanders (*Ambystoma*)

BY

GRANT A. MASON, JR.
JAMES L. HALL
PAUL GIBBONS ROOFE

Department of Anatomy, University of Kansas, Lawrence, Kansas

ABSTRACT

1. Twenty-two adult salamanders were used in this experiment, three for histological examinations, five for autonomic drug experiments, and the remainder for stimulation of the sectioned spinal cord.

2. The histological examination revealed nerves running among the smooth muscles of the acinus walls.

3. The autonomic drug experiments indicated that the nerves were from the sympathetic nervous system.

4. Stimulation of the sectioned spinal cord disclosed that the different levels of the thoracic and lumbar portions of the spinal cord (segments 10-15), when individually excited for a short period of time, would cause a segmental secretion on the tail.

5. Prolonged excitation of segments 10-15 will eventually cause secretion to appear on the entire dorsal surface of the tail, indicating the presence of a nerve syncitium.

6. This entire study proved that the walls of the poison skin glands in the tail of the salamander are innervated by sympathetic nerve fibers that have their cells of origin in spinal cord segments 10-15.

INTRODUCTION

The existence of "poison" skin glands in the tail of the salamander has been known for a great many years. According to Francis (1934), Leydig (1876), Pfitzner (1880), and Brasch (1894) were among the earliest known investigators to do research in regard to the development and histology of the "poison" glands. Zalesky (1866), also cited by Francis, was the first worker in the field to elucidate the chemical nature of the substance secreted by the

venom glands, indicating that there probably had been some work done on these skin glands and their secretion prior to his investigations. The above mentioned authors, along with more recent studies, Noble (1931), point out that the skin glands are of two kinds, mucous and poisonous.

However, an extensive review of the literature does not reveal the mechanisms involved in the activation of the glands and the exact manner of their innervation. A limited number of papers have been published on the autonomic nervous system of the salamander. Francis attributes to Andersson (1892), and Jaquet (1900), the first investigations in this area, the latter one being based upon the former and thereby suffering from some incompleteness found in the first work. Hoffmann's (1902) paper on the development of the sympathetic nervous system (sympathetic here referring to the autonomic nervous system as a whole) added several important details in this field.

The literature, since the early 1900's is lacking in references to these venom glands and their innervation. This paper deals with the relationship between the glandular structure and the autonomic nervous system.

This study was undertaken in order to determine the true nature, morphology, and innervation of the "poison" glands in the tail of the salamander. For this project the authors had available a limited number of adult salamanders, *Ambystoma tigrinum melanostictum* (Tiger Salamander) collected from the Jackson Hole area in Wyoming, and a large quantity of adult *Ambystoma annulatum* obtained through a biological supply house. Histological examination of the skin glands, gross dissection of the spinal cord, and injection of autonomic drugs, coupled with electrical stimulation, were made. The results indicate that the venom glands in the tail of the salamander are under the control of the sympathetic nervous system.

SURVEY OF THE LITERATURE

There is a scarcity of literature related to the "poison" skin glands and their direct innervation. Therefore this survey was conducted in two phases: one, that covering the glands of the skin, and two, that covering the autonomic nervous system of the salamander and that system's relationship to the innervation of the skin glands.

The literature dealing with the skin glands specifically is quite extensive. After the beginning of the 1900's there is a definite lack of pertinent references. The most recent reference that the

authors found was that of Francis (1934). This monograph, in regards to the skin glands and the autonomic nervous system, presents brief summaries of the more pertinent details found in many of the earlier works. The earliest report of these "poison" glands is by Ascherson (1840). Esterly (1902) says that Ascherson was the first to make the distinction of the two types of skin glands in an investigation of the glands of the live frog. It was Engleman (1872), however, who first used the terms 'Schleimdrusen' and 'Kornerdrusen,' thus recognizing the existence of the two types of glands. The literal translations of 'Schleimdrusen' and 'Kornerdrusen' are slime-forming gland and granular gland. This would seem to indicate that Englemann was using the word 'Schleimdrusen' to refer to mucous glands and the word 'Kornerdrusen' to signify venom or "poisonous" glands (serous glands). Most investigators share the opinion that there are two morphologically different types of glands yet the criteria for the differentiation has not always been the same.

The early classification such as non-contractile versus contractile, clear versus granular, or mucous versus poison were based on the difference in structure, physiological activity, and secretion. Dawson (1920) says that Englemann's 1872 classification is accepted to a degree. However, there is a diversity of opinion regarding the granular and poisonous glands. Dawson points out that Bruno (1904) classified the glands in *Rana esculenta* as holocrine and merocrine glands. It is not clear just what Bruno meant by this classification. Bruno probably was referring to the poisonous glands as being the holocrine type and the mucous glands as being the merocrine type. Physiological studies before and after Bruno as well as experimentation by Phisalix (1900), Hubbard (1903), and Shipley and Wislocki (1915) have proven conclusively that the skin of many amphibians secretes a poisonous substance. According to Phisalix, cited by Dawson, the poisonous glands are found not only on the dorsal surface, as most investigators had stated, but are also located on the ventral surface of the salamander. Previously, the majority of investigators who described the location and distribution of the poison glands stated that they are found in the dorsal skin only. In this case, dorsal refers to an area from the mid-lateral line of one side of the tail, over the dorsal aspect of the tail of the salamander, to the mid-lateral line on the other side of the tail.

Several investigators have supported the concept of one type of skin gland. The skin of *Proteus*, according to Bugnion (1873),

as cited by Dawson, has only one type of gland corresponding to the mucous type of Dawson. Bugnion's results were supported by Muhse (1909) and Phisalix (1912). The Muhse paper established this idea on a firm basis. She believes that there exists in the cutis of the toad, only one kind of gland. She goes on to say that the more recent classification into mucous or poison, nuclear or granular, is based on a difference in the epithelial structure and in the secretion produced. This view had also been supported by Englemann (1872), Seeck (1891), and Bristol and Bartelmez (1908). Several investigators, Calmels (1883), and Junius (1896), according to Muhse, have given evidence or expressed their belief in one kind of cutaneous gland. He has excluded from his discussion, the glands of the ventral surface, which are mucous. He makes the statement that the poison glands which occur only on the dorsal surface differ from the mucous glands of the ventral side, in that they contain a milky secretion produced by the poison cells of these glands. He makes no mention of smooth muscle fibers about the glands. It is probable from his description that he has seen the muscle fibers but has mistaken them for endothelial cells, which they resemble very closely.

All of the authors who described one type of gland believed that the mature form was the granular—poisonous type while the mucous gland should be regarded as an immature or younger stage of the poisonous gland. Calmels (1883) and Juninus (1896), mentioned above, are ardent advocates of this idea. The criteria for such a differentiation is usually morphological but Esterly (1902), who speaks of poison and mucous glands, uses the difference in the secretion as a basis for classification. In his work on *Plethodon,* Esterly says that the largest poison glands are situated on the back of the tail, lying in that portion of the skin covering the dorsal half of the tail. Esterly points out that the two types of glands are further distinguished by other features, chief of which is the staining reactions for mucous secretion, as previously reported by Nicogla (1893), and Hoyer (1890).

A firmer basis for differentiation can be afforded by a physiological classification. Shipley and Wislocki believe "that this work (physiological) has given, . . . added evidence for the separation of the cutaneous glands . . . into mucous and poison varieties." They say "the criteria for differentiation of the glands are usually morphological" but, as in the case of Briston and Bartelmez (1908), and Esterly (1902), who speak of the poisonous

and mucous glands, the difference in secretion has been used as a basis for classification. Physiological activity, assuredly, affords a firmer, more secure basis for classifying anatomical structures than a morphologic peculiarity, which often results from the technique used in handling the material studied. Shipley and Wislocki believed that their analysis had given added evidence for the separation of the cutaneous glands of at least one, possible two species of toad into mucous and poison varieties.

While the literature on the poison skin glands of various *Batrachians* is fairly extensive, Bristol and Bartelmez are apparently the only authors who have more recently dealt with the toad. Bristol and Bartelmez said in a short note in *Science* which deals with the toad but a great deal of which holds true for the salamander:

> The poison glands are found only on the upper surface of the body while the mucous glands are found all over the skin . . . They are much larger than the mucous glands and extend deep down into the compact corium layers. They are surrounded by a thin layer of loose connective tissue, which contains nerve fibers and a dense network of capillaries. There is almost a continuous layer of smooth muscle fibers about the gland.

Hubbard (1903) says that a macroscopic and microscopic examination reveals the anatomical nature of the swellings surrounding the glandular openings on the skin. However, he fails to follow this statement up and does not tell us what the anatomical nature is. He states that the dorsal half of the epidermis of this organ is covered with minute and thickly crowded pores which can be seen with the naked eye. Muhse indicates that the epidermis of the toad has so-called warts in the dorsal skin caused by the grouping of the glands. According to Noble, the glands are often of large size and clustered in pads as in the parotid glands of the common toad or in ridges, as along the back of many species of Rana.

Reese (1905) did considerable work on the Giant Salamander. He was not able to determine whether or not there were two types of glands present in the epidermis. He did state that there were three distinguishable regions, an epidermal layer, a fibrous layer, and a muscular layer.

Perhaps the best way to sum up the controversy concerning whether one or two types of glands exist, would be to quote Dawson who said:

> "Two types of quite different glands are found. There is nothing to indicate that the smaller glands are young forms in the development of the granular 'poison' type. No genetic relationship between the two types has

been found. The glands are of two distinct types that differ in development, histological structure, in the staining reaction of their secretion, and in their physiological activities."

The preponderance of authors seem to agree on the definitive structure of the poison glands. Esterly has one of the best descriptions of glandular morphology. He contends that a layer of contractile or smooth muscle fibers is found between the connective tissue layer and the glandular epithelis. These fibers were first shown by Hensche (1856). The muscles of the large glands are arranged in a single layer and have a general moridional direction on the gland, converging toward the upper pole. The fibers are usually simple but may be branched, this occurring mostly on the lower part of the gland. The muscles do not form a continuous sheet about the gland; the individual fibers are separated by spaces of greater or lesser extent. Esterly also says that he was not able to find with certainty, muscles on the glands which were mucous in nature. The existence of a sphincter or constrictor muscle for the glands has been claimed by Schultz (1889), quoted by Esterly, who described a band of muscle fibers running around the neck of the gland. This observation, however, has been contested by Drasch (1889). Esterly also was unable to find such a structure in *Plethodon.* However, both dilator and constrictor muscles occur about the mouths of the poison glands of *Plethodon.* The fact that the constrictor and dilator fibers lie entirely within the epidermis need not contend against their having the function of muscles, for according to Esterly, it has been well established that the intrinsic gland musculature has an ectodermal origin.

Reese holds the opinion that the poison glands are not surrounded by a muscular layer; that there is a fairly distinct basement membrane surrounding the epithelium of the gland; and the muscular layer which is sometimes described cannot be clearly seen. He disagrees with Drasch who reported in the salamander a complete layer of smooth muscle around the periphery of large poison glands.

There is good evidence in fish, chiefly in *Fundulus,* according to Spaeth (1916) that the melanophere may be a disguised type of smooth muscle cell. Morphological and embryological evidence does not permit a clear differentiation between melanophores and smooth muscle cells. Spaeth's physiological data is by far the most conclusive support of the parallelism between smooth muscle and melanophores.

Muhse, as previously noted, goes into great detail and is quite explicit about the glands. Her description of the wall of the

acinus is clearly stated. It is her belief that in some cases the wall of the acinus is thin and the muscle fibers form a more or less continuous sheath. Muhse says that Drasch is the only investigator who has made any reference to a substance which encloses the muscles fibers and on which rests the epithelia. The muscle fibers are elongated and spindle shaped. Several fibers are required to complete the circuit of the gland. There is, therefore, no definite arrangement of the nuclei about any given part of the acinus. Seeck (1891) describes similar structures, i. e. the spindle cells, but considers them replacement cells for the epithelia. He denies that they are muscles cells.

The innervation of the smooth muscles related to the poison glands is not well understood. Noble makes the general statement that the autonomic nervous system consists of a cranio-sacral or parasympathetic outflow and a thoraco-lumbar or sympathetic outflow. The extent to which the complex sympathetic responses of defense are organized in the amphibian as compared to the mammal is not clear. There is little reason to doubt that the bodily defenses in the amphibian are mobilized in accordance with our basic concepts of the autonomic nervous system.

It was pointed out by Francis that there are only three investigations published on the complete autonomic nervous system of the salamander, Andersson's, Jaquet's, and Hoffmann's. Andersson's arrangement is the most convenient to describe the system in three divisions: (*a*) cephalic; (*b*) cervical and abdominal; (*c*) caudal. The caudal portion, in which the present paper is primarily interested, consists of a double chain lying along the caudal artery. The ganglia are approximately segmental in arrangement. Connections between the two sides are fairly frequent.

The histological detail of the innervation of the glands was described by Esterly. Among the workers who have been interested in this problem are Canini (1883), Frenkel (1886), and Massie (1894). The innervation of the glands received less attention. Eckhard (1849) first demonstrated that the glands could be emptied by stimulating the anterior roots of the cerebrospinal nerves, but did not consider the structure of the nerve endings. Eberth (1869) reported that there was a network of very fine fibers close upon the glands and Openschowski (1882) described a network of nerves surrounding the glands as well as an intercellular net. Drasch (1889) also experimentally proved by nerve stimulation secretion can be obtained from the glands, as did Phisalix-Picot (1900). Loeb (1896) has also shown how closely the innervation of the

glands of *Ambystoma* are connected with the central nervous system. In 1898 Herrick and Coghill were able to show the existence of an intimate connection of nerve fibers with the walls of the glands, but were unable to discover the exact relationship of the fibers to the gland cells. They also described the plexus of nerves beneath the corium as being composed of two types of fibers; larger ones connected with the nerve bundles of the central nervous system, and smaller ones originating in ganglion cells in the corium. Schuberg (1903) has conflicting views, contending that many or all the nerve bundles described are really connective tissue bundles, and that the ganglion cells are the "Mastzellen" which he himself describes.

Concerning the work of Herrick and Coghill, Massie supports the arrangement of the fibers beneath the corium and also shows that the nerves end on the muscles of the "ental" glands. "Ental" is a word taken from Herrick and Coghill's work and connotes the poison type of gland. These authors found that the nerve fibers passing from the nerve bundle plexus under the corium are intimately connected with the ental glands, and seem distinct from the nerves supplying the muscles. The endings upon the smooth muscles are shown both by Cajal's silver method and Mallory's fuchsin stain, and in some cases are typical as described by Huber and DeWitt (1897) and Coghill (1899). In many cases fine branching fibers can clearly by seen lying upon the muscle layer. Two types of nerve endings have been described. Huber and DeWitt report that the fibers ultimately become delicate, slender twigs which, without terminal expansions, always lie on a muscle fiber and end there. Herrick and Coghill have contested that the muscles are supplied by nerves with typical endings of expansions or bulbs, as well as by fine twigs without terminal expansions. Their investigations also revealed the possibility of a connection between the nerves enveloping the glands and the gland cells, but they were not able to demonstrate it. Both the musculature and the epithelia of the granular glands have a direct nerve supply. The gland cells are surrounded by a basket work of fibers, which in some cases have terminal expansions lying on the nuclei.

Spaeth was previously cited as stating that the melanophores were possibly disguised smooth muscle cells. Concerning the innervation of melanophores, Spaeth has this to report; "as we know, the activity of vertebrate smooth muscle is normally controlled through fibers of the sympathetic nervous system. Voluntary

motor connections do not ordinarily occur." The innervation of the melanophores has been satisfactorily demonstrated histologically by Ballowitz (1893) and physiologically by Pouchet (1876) in several species of Teleosts. Satisfactory histological demonstrations of the nerve endings in the melanophores of amphibians and reptiles have not been recorded. Simmerman (1878) and Hooker (1912) have demonstrated physiologically the sympathetic innervation in the melanophores of the frog and Carlton (1904) has made similar observations in *Anolis*. Thus, the question of melanophores possibly being disguised smooth muscle cells seems to be unsettled.

Herrick and Coghill state that they succeeded in securing excellent preparations in the toad in which they find it easy to trace non-medullated fibers from the ectad plexus of the corium into the most intimate connection with the superficial walls of the glands. These glands are very large and quite active in the toad. The fibers are of small caliber, excessively numerous, and envelop the whole gland in what at first looked, to the two investigators, like a closely woven reticulum, but a closer study showed that the appearance was due to the repeated dichotomous branching of a large number of distinct nerve fibers. These fibers cross at slightly different levels and there was little doubt, in most cases, of the complete distinctness of the fibers as they crossed.

The pyridine-silver nitrate-pyrogallic acid method of Ranson gave Dawson the best results in staining nerve fibers. This method did not affect the sheaths but left the axis cylinder black. With this procedure, Dawson was able to observe nerves forming a wide mesh plexus in the subcutaneous connective tissue. Small bundles passed out from these plexi to the smooth muscle of the glands. He found, however, no evidence of the presence of a stratum of ganglion cells as did Herrick and Coghill, or Coghill himself. Dawson also disagrees with Esterly who found fibers which ran for a long distance under the dermis and eventually turned upward toward the epidermis. It was Dawson's belief that only free intercellular fibers were found in the epidermis. The fact that these fibers seldom branched and were never found to terminate in small knobs or plates has been described for many Amphibia. The distribution of the nerves to the glands was not apparent to Dawson because of their extreme fineness. Nor did he observe perinuclear baskets of nerve fibers, such as Esterly has described for the poison glands of *Plethodon*.

As mentioned previously in referring to Herrick and Coghill's paper, the glands in the skin of the frog are of two classes, distinguished as the ental and ectal series. The ental series refers to the poison glands while the ectal series designates the mucous glands. The glands of both series are distributed regularly throughout the skin of the head. The peripheral cells of the ectal series are approximately cubical, becoming lengthened or depressed according to the shape of the gland and their position in it. The walls of these glands are made up of such cells, supported externally by the fibers of connective tissue. There appears to be no differentiated muscular elements connected with these cells. The glands of the ental series have a peripheral structure that is entirely different. As with the ectal series, there is a tendency for the connective tissue to fold about them, but within this tissue, lying compactly on the surface of the gland, is a complete tunic of nonstriated muscle cells. On close examination it was discovered that there were a number of tunic cells sufficiently impregnated to show conclusively that the fibers within their varicosities lie, not between the cells, but upon them. Herrick and Coghill were unable to trace these fibers to an entrance into a bundle or as a single fiber for any distance beneath the corium, as was done in the case of the terminal fibers. The function of the fibers arising from the lower stratum, seems to be more intimately related to the glands of the ental series, for these fibers pass peripherally in large numbers and embrace the entire surface of each gland. These fibers do not seem to be identical with those described as innervating the tunic cells of the ental series. It seems that there are two groups of nerves passing to the glands of the ental series; one attaching by typical endings to the enveloping muscle cells, and the other ramifying profusely over the surface of the gland.

The nerve fibers were first described in 1862 by Kolliker as cited by Huber and DeWitt (1897). It is now believed that the endings on the involuntary muscle tissue are the neuroaxes of sympathetic neurons, situated in, or at some more remote point from the smooth muscle in which such endings are found.

Two very contradictory views have been expressed on the ultimate ending of these nerves in the involuntary muscle tissue. On the other hand, it is asserted positively that the nerve fibers terminate on the muscle cells, while on the other hand, some investigators find that the nerve fibers terminate in the muscle cell, on or in the nucleus. The results of Huber and DeWitt confirm

the observations of those writers who contend that the free ending of the terminal fibers end *on* the smooth muscle cells.

According to Hubbard (1903), some doubt has been cast on the theories of protection afforded these glands in the animal kingdom that have been in favor for half a century. The glands of *Plethodon oregonensis* secrete a milky fluid when the animal is stimulated by an induction current, either on the back or on the tail. In a similar manner the glands respond to the touch of a drop of acid or to irritation in the form of stroking with a knife blade. The secretion turns blue litmus paper red and is therefore thought to be acidic. It does not appear to be a mucoid as it is insoluble in water. *Diemyctylus torėsus,* when stimulated electrically, also yields a copious milky secretion, not merely upon the tail, but generally over the whole dorsal surface. *Batrachoseps attenuatus,* on the other hand, yielded very little secretion when stimulated in any like manner (Cope, 1889). From a comparison of the structure and the action of the glands of *Plethodon* with those of other *Batrachia* in which the nature of the secretion is known, Hubbard attributes to the secretion a poisonous and protective function. He judges that the tail glands in *Plethodon* offer a partial protection to the animal. They may perhaps by some offensive odor or by some irritating, volatile product, ward off an enemy.

Noble (1931) postulates that the poison glands protect their owner from being devoured by many possible enemies. But these defensive properties do not always protect toads and salamanders from being eaten by snakes and other Amphibia. Three alkaloids have been extracted from the poison; Samandrin—$C_{26}H_{40}N_{20}$—being the principle one and affecting the respiratory area of the central nervous system of the predator.

Since the nature of the secretion and the function of the glands is not the primary objective of this paper, little more will be said about these two subjects. The purpose of the present investigation is to firmly establish the source of innervation of these venom glands and to determine the manner in which the nerve fibers terminated in relation to these glands.

MATERIALS AND METHODS

Nineteen live adult salamander, five *Ambystoma tigrinum melanostictum,* and fourteen *Ambystoma annulatum* were used with three previously formalin fixed adult species of *melanostictum* to conduct this study. All *melanostictum* were obtained by collection

in the Jackson Hole area of Wyoming. After collection, these animals were transported to Lawrence, Kansas, where these experimental procedures were performed. The *annulatum* used in this study were procured through a biological supply house. Bishop's *Handbook of Salamanders* (1943) lists the distribution of *annulatum* as "vicinity of Hot Springs, Arkansas, and Stone County, Missouri" and *melanostictum* as "British Columbia, Alberta, Washington, Oregon, Idaho, Montana, Wyoming, North Dakota, South Dakota, and Nebraska."

All of the animals, prior to experimentation, appeared to be physiologically normal. The average weight of *melanostictum* was 56.1 grams and that of *annulatum* was 19.6 grams. The three previously fixed adults, mentioned above, were sacrificed in Moran, Wyoming at the Jackson Hole Biological Research Station and also transported to Lawrence, Kansas, for further investigation.

Each animal, preliminary to any investigation, was injected intermuscularly with a paralyzing drug, *Intocostrin* (E. R. Squibb and Sons, New York, New York). *Intocostrin* is an aqueous solution obtained from purified *Chondodrendron tomentosum* extract. The drug is believed to have an affect similar to that of a poison called curare (curari) used as an arrow poison by South American Indians. Its paralyzing action is reported to act upon the myoneural junction. This drug was used because it afforded normal physiological results without interfering with the action of the autonomic nervous system.

The first five animals, *Ambystoma tigrinum melanostictum,* were injected with 0.30 cubic centimeters of *Intocostrin;* approximately 0.15 cubic centimeters in both the dorsal pelvic and dorsal pectoral musculature. Fifteen minutes after this injection each animal was placed on its back. It was to establish whether or not the animal was anesthetized by pinching one of the animal's digits, to confirm the level of the withdrawal reflex. The skin covering the ventral aspect of the pectoral girdle was stripped away. Each of the two overlapping cartilaginous portions of the pectoral girdle was reflected laterally and held in that position with a hemostat. This operative procedure exposed the pericardium through which could be seen the beating heart. The thin pericardium was also removed, completely exposing the heart.

By means of a 1.0 cubic centimeter tuberculin syringe and a 30 gauge, ¾ inch hypodermic needle the different chemicals used in the experiment were injected into the heart.

AUTONOMIC DRUGS AND STIMULATION

Animal Number 1: Approximately 0.05 cubic centimeter of Adrenalin in 0.9% saline solution was injected into the ventricular cavity of the heart. A thirty second resting period followed the injection. Immediately ensuing this thirty second latent period a shock from an electronic stimulator, Model 751, 115 volts AG, 50-60 was administered via insulated electrodes to the dorsal portion of the tail at the spinal cord levels of S-1 through S-3. The stimulus has a frequency—pulse per second of twenty, a duration of ten milliseconds, and a voltage of three. At all times during the stimulation the tail was closely observed with the naked eye for any evidence of secretion. After obtaining the results of this experiment and allowing a sufficient amount of time for the glands to recover, the following four chemicals were also injected individually, in the same manner and quantity, into the heart. The chemicals were Prostigmine and Acetylcholine, Atropine, and Hexamethonium bromide. These were introduced individually and followed by stimulation. The chemicals, including Adrenalin, were used in a dilution of one part to one thousand parts of distilled water. The dosage was 1.0 milliliter per kilogram of body weight. A stimulation of the same intensity and frequency was applied in a similar manner and location in the case of each drug. A resting period of fifteen minutes was permitted after each phase (different chemical) of the experiment. This animal was sacrificed to be used in a staining technique for peripheral nerves by Williams (1943) whose report stated that he had obtained excellent results with amphibians. The procedure involved the macerating of tissue in KCN and immersing in Ehrlich's Hematoxylin.

Animals Numbers 2-5: The procedures of anesthetization, exposure of the heart, injection of the chemicals, stimulation, and observation used on animals number 2 through number 5 were identical to those used on animal number 1. The mode of procedure varied, however, in the number of chemicals used. Animals number 2 through number 5 had individual injections of Atropine, Adrenalin, Hexamethonium bromide, Prostigmine and Acetylcholine as well as another chemical, Ergotoxine ethane sulfonate. After the injection of Ergotoxine ethane sulfonate and normal stimulation, the voltage was increased to seven and the frequency raised to seventy-five and the animal was once again stimulated.

STIMULATION OF SECTIONED SPINAL CORD

Animals Numbers 6-12: This group of seven *Ambystoma annulatum* could not be considered to be a definitive experiment in themselves. They did, however, lay the background for the seven following animals and the experimentation performed on them. Each of the animals in this group was used to perfect a technique or techniques that were to be used later. The dorsal musculature covering the vertebral column was removed and the vertebra were opened to expose the spinal cord. The objective was to ascertain what sections of the spinal cord would elicit a secretion when stimulated. The cord was sectioned at various levels. The tail skin was observed when a stimulus was applied above and below the level of sectioning.

Animals Numbers 13-19: A similar procedure, as will be mentioned below, was used in this step of the investigation for each of the seven *Ambystoma annulatum.* All animals in this group received an injection of Intocostrin in the dorsal pelvic and dorsal pectoral musculature according to their weights, *i. e.,* 0.3 cubic centimeters per fifty grams of body weight. Under curare, the skin on the dorsal side of the thorax, half an inch on either side of the spinal column, from thoracic vertebra 1 to thoracic vertebra 15 was removed. It has been reported that the number of thoracic vertebra are not constant and can vary from thirteen to fifteen. Our study indicates only fifteen. The underlying thin spinal musculature was also removed. This step exposed the bony spinal vertebra. The spines and the dorsal half of the body of each vertebra were carefully dissected away, leaving the bare spinal cord lying in a trough created by the ventral portion of the thoracic vertebra.

The final step in this phase of the investigation was to individually sever each segment and stimulate it above the point of transection, observing grossly the reactions of the glands in the tail. Between severing and stimulation there was an interval of time to allow for recovery from the shock of the operation. The average time of the recovery period was fifteen minutes. The voltage of the stimulus was three, the frequency was twenty, and the duration was ten milliseconds. Each cord segment received a shock that had a length of two seconds. A twenty minute resting state followed each of the segmental stimulations.

Then, each segment that caused an initial secretion on the tail was again stimulated. The current was maintained on these segments until there was secretion over the entire tail or until it was evident that no matter the length or strength of the stimulus, no

secretion would be brought forth. This procedure gave a great deal more insight into the nerve distribution in the tail of the salamander. This procedure was adopted as a step in the investigation for the reason that a short period of stimulation on some of the caudal segments (12, 13, 14, and 15) of the thoracic cord would not cause secretion over the entire dorsal surface of the tail.

SECTIONING AND STAINING THE TAIL

Animals Numbers 20-22: The tails of these three animals, *Ambystoma tigrinum melanostictum,* were used to prepare the histological specimens required by this investigation.

Number 20: Immediately after death, this animal was placed in a 10% formalin solution where it remained until studied. When the animal was removed from the formalin its tail was severed from the body and prepared for dehydration. Among the necessary steps in the preparation was the removal of the vertebral column. The column was removed by placing an incision on the "nape" or extreme dorsal portion of the tail and one at the most ventral portion of the tail. These two incisions were continued toward each other, both splitting and going around the bony structure and meeting their fellow from the opposite side. Thus we had two pieces of tissue, a left and a right side of the tail. The amphibian skin was then dehydrated, embedded in paraffin, and serially sectioned at fifteen micra. Every tenth section was mounted and stained with Hematoxylin and Rosin.

Number 21: The same procedure as above was used except that the vertebral column was removed in such a way that the tail became a hollow cylinder permitting free access of the fluids to all its parts. The skin was stained with a modified Margolis-Pickett (1956) Luxol Fast Blue—Oil Red O method.

Number 22: The tail was cut into small sections, 10 mm. in length, after the entire animal had been fixed in 10% formalin. These tail sections were decalcified in a solution consisting of 10% nitric acid in 70% ethyl alcohol. The usual dehydration methods were used. Serial sections in paraffin were then impregnated with a modified Holmes silver technique.

RESULTS

AUTONOMIC DRUGS AND STIMULATION

Animal Number 1: Following the injection of 0.05 cubic centimeters of Adrenalin in 0.9% saline, there was an immediate doubling

of the cardiac rate to ten beats per five seconds. The electrodes were applied to the dorsal nape of the tail. When the current was turned on, the poison glands released a copius, white, sticky secretion within two seconds. The same shock was applied to the ventral regions of the tail but there was no evidence of any secretion.

Prostigmine, followed shortly (thirty seconds) by Acetylcholine was injected into the heart in quantities of 0.05 cubic centimeters. The second injection caused a primary cardiac acceleration which was soon altered to an over-all deceleration of three beats every five seconds. Similar to the Adrenalin injection, there was a slight watery appearance on the ventral side of the tail.

The third injection was Atropine. The procedures relating to quantity of the drug, injection, the waiting period, the stimulation, and observation were identical to those used in the Adrenalin and Prostigmine-Acetylcholine experiments. The results with this drug were very similar to those obtained when using the three perviously mentioned compounds. There was an immediate and copious secretion on the dorsal surface of the tail when it was stimulated. In this case there was no evidence of a watery secretion on the ventral surface.

Hexamethonium bromide was the fourth and final chemical used on animal number 1. There was none to very slight secretion on any portion of the tail when it was electrically stimulated. The only part of the tail that showed any evidence of secretion was the tip.

Animals Numbers 2-5: The four autonomic drugs used on animal number 1 were also used on animals numbers 2-5. The methods of experimentation were the same and the results were also identical. See Table 1. However, a fifth drug was introduced into these four animals.

The fifth chemical was Ergotoxine ethane sulfonate. It was injected in a quantity similar to that previously used on all the animals. The heart had almost an immediate slowing reaction. The rate was two beats per five seconds but each contraction was violent. In five minutes there was a distinctive blanching of the heart and the beats had slowed to one per five seconds. There was no evidence of any secretion elicited by stimulation other than on the last eighth of an inch of the tail. Even though the voltage and frequency as well as the length of stimulation were all significantly increased, no secretion was seen to come from the poison glands.

STIMULATION OF SECTIONED SPINAL CORD

Animals Numbers 13-19: Of the seven animals used in this step of the experiment, no secretion was ever observed when thoracic cord segments one through nine inclusive were stimulated. There was, however, a gradient found in segments ten through fifteen relating to the amount of secretion that came forth when the respective segments were stimulated. See Table II. Segment ten, when stimulated, caused the entire dorsal half of the tail to secrete. Segment eleven, like segment ten, also caused the entire dorsal portion of the tail to secrete. Segments twelve through fifteen however, caused a segmental secretion on the tail when stimulated. To clarify the above, we should note that segment twelve caused the caudal two-thirds of the tail to secrete, segment thirteen caused the caudal one-half of the tail to secrete, segment fourteen gave rise to secretion on the last one-third of the tail, and segment fifteen gave rise to secretion on only the very minutest portion of the tip of the tail.

The secretion, caused by stimulation of thoracic cord segments ten through fifteen, had only a slight delay from the moment the electrodes contacted the cord until the milky white fluid was seen. It was observed that stimulation of segments ten and eleven caused a secretion to appear over the entire tail. The secretory product, however, did not appear simultaneously over the entire dorsal surface. Here too, there was a step-wise gradient, the cephalic portion of the tail secreting first and a wave of secretion appearing to spread down the tail. The entire sequence, from the moment of stimulation until the dorsal half of the tail was covered with the secretion, took less than two seconds.

The most interesting results were found when the stimulation was continued over a period of time exceeding two seconds. Stimulation of segments ten and eleven was not necessary in this step for it had been found by previous experimentation that both of these levels gave secretion over the entire tail in less than two seconds. It was found that a continual stimulation of thoracic segments twelve through fifteen would eventually cause all of the poison glands on the dorsal portion of the tail to secrete. As one stimulated progressively down the cord, the longer the time lapse before the entire tail was covered with the poisonous secretion. Table II shows the length of time necessary for stimulation of the various cord segments before the secretion would appear. Seven

salamanders were used to compute the average time. *Ambystoma annulatum* was used to calculate the segmental times. Using ten specimens of A. tigrinum melanosticum with average weights (25 grams) a similar pattern of secretion was obtained in preliminary testing at the Jackson Hole Biological Station in the summer of 1960 (Roofe and Mason).

THE SECTIONED AND STAINED TAIL

Animals Numbers 20-22: A histological examination of the poison gland cells was made utilizing two different types of staining techniques. The Luxol Fast Blue—Oil Red O method did not give clear cut results. Therefore, this study was limited to those slides stained with hematoxylin and eosin and Holmes silver stain. There is a single layer of smooth muscle fibers forming a continuous sheath or thin wall around the body of the gland. This sheath was barely thicker than two muscle fibers and this condition was infrequently noted and found only when one fiber overlapped another. A majority of the walls of the poison glands were one cell thick. Thin nerve fiber endings were found lying in intimate contact with these smooth muscle fibers. This observation confirmed the very early work of Hensche.

The normal poison gland cells appeared to contain three different types of secretion. There appears to be a gradient in the secretion's relationship to the surface opening of the gland. There is a mass of large, ovoid, cellular bodies close to the surface opening, occupying less than one-fourth of the total area of the gland. In the central two-thirds to three-fourths of the gland is another glandular mass composed of smaller spherical cells. The remainder of the secretion found within the unstimulated cell had the appearance of "protein" material, having no cellular outline and resembling a homogeneous mass.

All of the poison glands of the salamander, regardless of their volume, retained their ovoid or spherical shape.

A thorough survey of all the slides made failed to reveal any type of glandular epithelia. Several of the more prominent authors of the late 1800's, including Esterly, spoke of this epithelium.

DISCUSSION

AUTONOMIC DRUGS AND STIMULATION

The drugs employed in this experiment were chosen because of their single direct effect. Adrenalin potentiates adenergic nerve

endings and it was felt, that by use of this drug, if the glands were under sympathetic control, they would release an even greater quantity of secretion than the normal amount. However, there was no device to quantitatively measure the amount of secretion under the normal or experimental conditions and the glands seemed to give less secretion every time they were stimulated. Therefore, the resting period was introduced. This resting period apparently enabled the poison glands to replenish their secretion. It had been found that continual stimulation would exhaust the glands so that they would have little or no secretion remaining in them after several prolonged shocks.

Prostigmine and Acetylcholine, on the other hand, mimicked the action of the parasympathetic nervous system by potentiating cholinergic nerve endings. Prostigmine destroyed Cholinesterase which itself destroys Acetylcholine. Thus, when the Acetylcholine was introduced after the Cholinesterase had been destroyed, we had an increased action of the parasympathetic nervous system without disrupting the action of the sympathetic system. Once again the animal was stimulated and we had a copious secretion. This experiment in itself was not conclusive enough to prove whether the glands were under control of the sympathetic or parasympathetic nervous system. The watery secretion on the ventral surface of the tail, mentioned in the results, was from the numerous mucous glands covering the body. A discharge of mucous can be obtained by handling or a slight mechanical stimulation.

The third drug used was Atropine. This chemical blocked the cholinergic nerve endings. Therefore, in the way of negative proof, the copious secretion we obtained upon stimulation would tend to prove that the poison glands are under the control of the sympathetic nervous system. In other words, if there was a copious secretion when the parasympathetic nerve endings were blocked, the conclusion that the glands were under adenergic (sympathetic) control would be supported.

To be sure that the glands were under a nervous control simulated by the autonomic nervous system, Hexamethonium bromide was injected. This drug blocks synaptic transmission at the ganglion thus producing a total block of all autonomic nerve endings. Thus, when a stimulus was applied, there should be no evidence of any secretion. This drug was injected in order to determine whether the adrenal glands were causing the skin glands to be activated. If the adrenals were affecting the activity of the poison glands

we would expect a secretion in spite of the drug injection. When the stimulus was applied there was no evidence of any secretion, indicating two things. One, the glands were autonomically controlled and two, the adrenals had nothing to do with the secretion.

However, as previously stated, we had only negative evidence that the poison glands were controlled by the sympathetic nervous system. It was thought, therefore, that his previous set of experiments should be carried one step further. A drug that blocked the adenergic nerve endings was needed. Ergotoxine ethane sulfonate was chosen. Because of inconclusive data obtained from animal number 1, it was thought that this animal should not be included in Table I.

With all five different drugs at hand, four separate tests were performed. The results of these tests have been tabulated in Table I. One can see that every test, when repeated, gave results identical to those of the previous experiment. Likewise, the four experiments that were performed upon animal number 1, when repeated on animals number 2 through number 5, gave results similar to those found in the first animal. The fifth drug in Table I is Ergotoxine ethane sulfonate. The results with this drug support the contention that the poison glands are under sympathetic nervous control. Since there was no secretion on stimulation, the most obvious conclusion is that these glands are under autonomic nervous control and specifically, more the sympathetic nervous system. Ergotoxine had to be injected last because its affect was permanent in the dosage in which it was administered. In addition to this reason, the drug does not easily oxidize and would therefore remain in the system a long time, regardless of the dosage. The blanching of the heart, after the injection of Ergotoxine, was due to the imbalanced activity of the parasympathetic nervous system. The adenergic nerve endings, primarily the sympathetics on the coronary vessels, were blocked, causing an antagonistic constriction of these vessels and therefore blanching.

There might be objection to the previous statement that upon stimulation after an injection of Ergotoxine, "there was no evidence of any secretion other than the last quarter inch of the tail." This statement can be better understood if we realise that the circulation in the caudal quarter inch of the tail is quite poor. This could explain the slight secretion found when Ergotoxine had been introduced and the tail stimulated. Since the drug must reach the nerve endings by diffusion through the blood vessels and capillaries

and their distribution in the area is poor, it stands to reason that some of the nerve endings would not receive any Ergotoxine and therefore remain quite effective.

Williams (1943) method of staining peripheral nerves in cleared vertebrate tissue was attempted on animals one and two. The results of this procedure were not fruitful enough to permit the continuation of this method. The principal difficulty was the extreme fineness of the nerves thereby making it impossible to follow their course shortly after they left the spinal cord.

Stimulation of the Sectioned Spinal Cord.

Since it was determined that the glands of the tail were under sympathetic control, an attempt was made to determine the level of the spinal cord from which the fibers innervating these glands originated. Before this part of the experiment was begun nothing in the way of segmentation of the secretion was observed when the tail was grossly stimulated.

As there was some quantity of secretion whenever thoracic segments ten through fifteen were stimulated, we can say that the sympathetic nerves running to the walls of the poison glands have their cells of origin in these six thoracic cord segments. We can also state, as Table II indicates, that there is a segmental arrangement of these fibers with considerable overlap. This segmental arrangement and overlap have been pointed out in the results.

Stimulation of the more caudal thoracic cord segments (12, 13, 14, and 15) for a period longer than two seconds caused the entire tail to be covered with secretion. This lead to some speculation. At first it was believed that the stimulatory messages were being conveyed to the brain, primarily the hypothalamus, and then relayed back down to the poison glands. This, of course, would cause the slight delay noted when the electrodes are placed on the cord until the secretion appeared. Yet, this would appear to be difficult because most every known pathway that traversed back to the brain had been severed. The only logical conclusion that one is able to arrive at is that we have a local reflex arc and that there is a nerve syncitium that spreads throughout the entire tail. This syncitial arrangement would account for the slight delay before the tail is covered with the secretory product, as well as solve the problem that had arisen when all the pathways back to the hypothalamus and other autonomic regulatory areas had been severed. This syncitial net would also solve the problem of the wave of

secretion spreading up the tail when segments 12, 13, 14, and 15 were continually stimulated, rather than spreading down the tail as it would if the nervous messages were coming from the brain.

THE SECTIONED AND STAINED TAIL

Francis, when speaking of the autonomic nervous system, says that there are no special features to note other than the fairly frequent anastomoses between the two sides. He devotes considerable space in his monograph to the venom glands and does not mention the manner in which the glands are innervated. He completely ignores the thesis of glandular innervation.

When investigating the histological composition of the poison glands, the concepts of Spaeth were borne in mind. He thought that there was a good possibility that the melanophores could be disguised smooth muscle cells. We could find no good histological evidence that they (the melanophores) were directly innervated by the sympathetic nervous system. The melanophores, themselves, were easily identified as were the darkly staining fibers that ran through and among the muscular walls of the glands. Yet it could not be demonstrated satisfactorily to us that the nerve fibers actually terminated on the melanophores. The fibers passed close to the pigment granules but in no cases were knobs, boutons, or spindly endings seen to terminate upon these pigment granules.

Huber and DeWitt brought up the controversy of the true endings of the nerve fibers. They expressed the idea that two very contradictory views had been put forth; that nerve fibers may terminate in relation to the spindle shaped muscle cells or on or in the nucleus of the smooth muscle cell. We agree with Huber and DeWitt; the nerve terminals are *on* the muscle cells and in no case was it found that the free endings were on or in the nucleus of the muscle cell. This evidence corresponds to the work of Herrick and Coghill in 1898 who were able to show the existence of an intimate connection of the nerve fibers with the walls of the glands.

Dawson and several other investigators felt that there was definite epithelial layers associated with the wall of the gland. However, very close observation did not reveal any structure that resembled an epithelial layer. The difference in this particular part of the glandular morphology is very likely due to the fact that Dawson's investigations were on *Plethodon* while the present work is based on *Ambystoma tigrinum melanostictum.*

We modified the histological techniques to better suit the tissue at hand. A thorough examination of the first preparations revealed that too much detail had either been lost or was not able to be observed. Both the staining technique, Hematoxylin and Eosin, and the method of removal of the vertebral column were modified as described in the materials and methods.

The two modifications of the silver staining method mentioned in the materials and methods dealing with animal number 15 are as follows. The modification involved a shortening of several of the important steps (cutting the time in the impregnating solution from twenty-four hours to twenty-two hours and reducing the time in the reducing solution from two to five hours to one and one-half hours). This shortening seemed to stain the sections more in the manner desired, *i. e.*, more contrast. The original time sequence made the slides too dark and very little structure in the way of nerve fibers was discernible.

Our observations concerning the volume of the glands and thereby their shape (ovoid or spherical) followed the reports of Dawson, but were contrary to the ideas and writings of Drasch, Nierenstein (1908), Muhse, and Shipley and Wislocki.

THE REASON FOR AND MANNER OF THE SECRETION

The skin of the salamander is moist and devoid of scales. It is highly glandular and contains two different types of glands, the mucous glands, whose function it is believed, is to keep the skin moist, and poison or venom glands whose function will be speculated upon briefly. The manner of expulsion of the poison and its true chemical nature is somewhat of a mystery. When a salamander is irritated by chloroform, the white, milky fluid is seen to exude from the venom glands. Likewise, Dawson has said that the "granular secretion is expelled only in responses to some violent stimuli either mechanical, chemical, or electrical." He also believed that the secretion of the poison was due to the contraction of the smooth muscles found about the gland sacs. Seeck says that the expulsion is caused by underlying skeletal muscle. This idea of Seeck's can not be held as true because the primary injection of *Intecostrin* blocked all of the skeletal myo-neural junctions, rendering the skeletal muscle incapable of any response. Actually, later authors quite generally agreed that the expulsion of the secretion was due to smooth muscle contractions. Yet Calmels, like Seeck, did not attribute expulsion to the smooth muscle fibers about the

individual glands. Calmels did not describe or diagram, as such, a layer of smooth muscle fibers in the acinus wall. His statements regarding muscles are not clear, and Muhse felt that it was best not to attempt to state his position.

The secretion of the mature glands comes to the surface of the skin in drops. It is white or creamy in color, has a bitter taste, and a rather strong, disagreeable odor, very similar to that of Jimsom weed (rank-smelling foliage). Inside the mature glandular cell, the secretion as mentioned before, is primarily granular in appearance and takes stain readily, so that it always has a decided color, thus easily distinguished from the mucous secretions which are only very slightly affected by stains.

Noble reported that these granular glands produce a secretion which is usually more toxic than that of the mucous glands. It is believed that the secretion contains more than one poisonous constituent. Faust (1898), according to Noble, isolated Bufotalin, giving it the empirical formula of $C_{34}H_{46}O_{10}$. Francis states that Zalesky isolated the chlorohydrate of one of the alkaloids of the poison, which he called Samandrin. Abel and Macht (1911), in analyzing the secretion of the tropical toad, *Bufo agus*, said that the glands are under the control of the central nervous system.

Bristol and Bartelmez verified that the secretion had the reputation of being poisonous. They reported that it acted only on the mucous membrane, producing a result similar to that of curare.

CONCLUSIONS

In order to better understand the poison skin glands of the salamander under normal and stimulatory conditions, histological examinations, stimulatory experiments coupled with sectioning of the spinal cord, and autonomic drug injections followed by stimulation, were made on the tails of twenty-two adult salamanders.

We found that the walls of each of the poison skin glands was surrounded by a thin layer (one cell thick) of smooth muscle. In contrast to these walls, the mucous glands contained no smooth muscle in their walls.

In close and intimate contact with the smooth muscle cells of the walls are tiny, slender nerve endings. These fibers are from the sympathetic nervous system and come from thoracic cord segments ten through and including fifteen.

By the use of autonomic drugs and stimulation we found that the poison glands in the dorsal portion of the tail are innervated

TABLE I.—Secretion Results

CHEMICAL	Animal I	Animal II	Animal III	Animal IV
Atropine	Copius	Copius	Copius	Copius
Adrenalin	Copius	Copius	Copius	Copius
Hexamethonium bromide	Nothing	Nothing	Nothing	Nothing
Prostigmine and Acetylcholine	Copius	Copius	Copius	Copius
Ergotoxine ethane Sulfonate	Nothing	Nothing	Nothing	Nothing

TABLE II.

Thoracic cord segments	Results of two second stimulation of severed cord segments	Length of stimulation necessary to cause entire tail to secrete
1	Nothing	—
2	Nothing	—
3	Nothing	—
4	Nothing	—
5	Nothing	—
6	Nothing	—
7	Nothing	—
8	Nothing	—
9	Nothing	—
10	Secretion over entire dorsal half of tail	1.4 seconds
11	Secretion over entire dorsal half of tail	1.8 seconds
12	Secretion over last two-thirds of tail	2.5 seconds
13	Secretion over last one-half of tail	2.8 seconds
14	Secretion over last one-third of tail	3.5 seconds
15	Secretion only on the very tip of tail	4.1 seconds

in a serial fashion. That is, the last thoracic segment of the spinal cord, when stimulated, would cause a secretion to appear on the very last portion or tip of the tail. Whereas a more anterior segment, when stimulated, would cause secretion over a greater portion of the tail.

The total innervation of the poison glands is bound up in a nerve syncitium. This accounts for the wave of secretion being able to spread either way on the tail, the delay in the appearance of secretion over the entire tail, and the manner in which the glands are innervated when all known pathways back to the brain have been severed.

These glands appear to be protective in nature for the secretion is only released when the animal is severely traumatized.

SUMMARY

1. Twenty-two adult salamanders were used in this experiment, three for histological examinations, five for autonomic drug experiments, and the remainder for stimulation of the sectioned spinal cord.
2. The histological examination revealed nerves running among the smooth muscles of the acinus walls.
3. The autonomic drug experiments indicated that the nerves were from the sympathetic nervous system.
4. Stimulation of the sectioned spinal cord disclosed that the different levels of the thoracic and lumbar portions of the spinal cord (segments 10-15), when individually excited for a short period of time, would cause a segmental secretion on the tail.
5. Prolonged excitation of segments 10-15 will eventually cause secretion to appear on the entire dorsal surface of the tail, indicating the presence of a nerve syncitium.
6. This entire study proved that the walls of the poison skin glands in the tail of the salamander are innervated by sympathetic nerve fibers that have their cells of origin in spinal cord segments 10-15.

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